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Molecular Characterization and Phylogenetics in the *Rhizophora* Species Complex at Pichavaram, Tamil Nadu: A Potential Resource for Phyto-Pharmacology

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Abstract: Mangroves are spatially limited bioresources known for their potential pharmacological uses. The genus *Rhizophora* is a conspicuous one reported to have enormous biopotentials in treating inflammation, diabetes, and rheumatism. However, mangroves are recently experiencing severe threats due to the over-exploitation of valuable tannin resources, climate change, and other anthropogenic pressures. *R* × *annamalayana,* a natural hybrid in Pichavaram, is rare in its distribution and a source of bioactive compounds with anticancer properties. The pure nature of the hybrid makes it more vulnerable to extinction. Therefore, domestication, conservation, and sustainable use of valuable resources are needed on a date. Poor genetic structure in mangroves is reported to be one of the reasons for extinction. Hence, understanding the population genetic structure of the genus is of paramount importance. Therefore, the present study aimed to understand the population genetic structure of the *Rhizophora* species complex in Pichavaram with the following objectives, to carry out the molecular characterization of the putative hybrid and to examine the phylogenetic relationship to its parental species using microsatellite markers. The study identified that putative hybrid *R.* × *annamalayana* (mean H_E = 0.592) has more significant variability than the putative parents *R. apiculata* (mean H_E 0.611) and *R. mucronata* (mean H_E = 0.667). The negative inbreeding coefficient value or Wright's Index in *R.* × *annamalayana* (- 0.190) suggests ample variation among the putative hybrids. The putative hybrid is genetically more proximal to *R. apiculata* than *R. mucronate*. Phylogenetic studies indicate that ten samples out of fifteen were clustered with *R. mucronata*, and the rest five grouped with *R. apiculata* indicating the varying paternal and maternal combinations.

Keywords: Inbreeding Coefficient, Nei's Distance, Phylogenetics, and Wright's Index.

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1. INTRODUCTION

Mangroves are ecologically essential bioresources with pharmacologically valuable flora distributed in the intertidal regions of the tropical and subtropical zone ($30^{\circ}N - 30^{\circ}E$). This group of plants is facultative halophytes, adapted to the muddy, anaerobic environment, extreme temperatures, and tidal regimes[1]. These physiological adaptive mechanisms allow these plants to metabolize various active compounds that provide resilience in adverse environments[2]. These active compounds play a significant role in the treatment of human illness. Most mangrove plants are widely used as traditional therapeutics[9]. In addition, mangroves provide tangible products to millions of life systems in and around tropical countries[3]. Mangroves are also known for their numerous ecosystem services, carbon sequestration, and coastal protection [4,5]. Globally, eighty-one mangrove species are recorded under thirty genera and seventy families[6]. *Rhizophora* is a frequently noticeable genus in most of the mangrove realm with enormous biopotentials to treat inflammation, diabetes, and rheumatism[7]. In India, the genus *Rhizophora* is represented by three species (*R. apiculata*, *R. mucronat*a, and *R. stylosa*) and two putative hybrids (*R.* × *annamalayana* and *R.* × *lamarckii*). Earlier investigations demonstrated the Anti-HIV activities of *R. apiculata*, the anti-inflammatory and antimicrobial properties of *R. mucronata,* and the hybrid *Rhizophora* × *annamalayana*[8, 9]. Despite this, the genus *Rhizophora* is reported to produce high levels of tannin that are a potential source for free radical scavenging activity [10, 11]. The putative hybrid *Rhizophora* × *annamalayana* occurs in Pichavaram (Tamil Nadu), a deltaic mangrove habitat from the Indian East Coast[12]. The hybrid nature and parentage of this species were initially described by Kathiresan[13], based on its intermediate morphology to the putative parents[14]. Later several investigations confirmed the hybridity of the species using molecular and DNA-based tools[15-19]. The hybrids are gigantic, with more excellent growth rates than their parental species[12,20], which promises a sustainable source of bioactive metabolites. However, the hybrid *R* × *annamalayana* is rare within Pichavaram. New regeneration of the said hybrid is extremely rare[21]. The taxon is male-sterile; however, known to produce propagules (very rarely) that do not grow into individual trees[22]. The sterile nature of the hybrids makes them more vulnerable[15]. The study location Pichavaram is a RAMSAR site. Recently, it has been subjected to several climate change extremities (*Thane* - 2011, *Vardah* - 2016, *Okchi* – 2017, *Gaja* - 2018, etc.). The infrequent population of *R.* × *annamalayana* in this location desires conservation and domestication for the sustainable extraction of biopotential supplies. Adequate protection in any mangrove resources requires a complete understanding of genetic diversity, as it is a fundamental component to accessing biodiversity[23]. Knowledge of genetic diversity and patterns in threatened populations is critical to determining their health status, survival into posterity, and long-term endurance [24]. Both natural and anthropogenic factors generally determine the genetic diversity pattern among the population of taxa. Therefore, it is a prerequisite to generate empirical data on these patterns. Similarly, lesser considerations on this aspect could also hamper the conservation effects[17]. Thus, the present study aimed to understand the population genetic structure of *the Rhizophora* species complex in Pichavaram with the following objectives, to carry out the molecular characterization of the putative hybrid and to examine the phylogenetic relationship to its parental species using microsatellite markers.

2. MATERIALS AND METHODS

2.1 Study Site and Target Taxa

Field surveys were conducted at the study site Pichavaram (11°17'- 11°30'N; 79°45'-79°50'E), a deltaic mangrove habitat of the Indian East coast. Plant collections were made in all three *Rhizophora* species. Specimens were initially identified using the flora. Three specimens in each taxa were sampled and deposited in Fisher's Herbarium at the Institute of Forest Genetics and Tree Breeding, Coimbatore (Table 1). This study sampled fifteen *Rhizophora* × *annamalayana* accessions and three accessions in each parental species (Table 2).

Table 1. Details of Accessions submitted to the Fisher's Herbarium, Forest campus, Coimbatore, TN.

S. No	Species	Herbarium ID	Geo-specifics
1	*R. apiculata* Blume.	FRC 25050	11°25'73.0" N; 79°47'62.2" E
2		FRC 25051	11°26'39.2" N; 79°47'55.8" E
4	*R. mucronata* Poir.	FRC 25048	11°25'55.9" N; 79°47'36.4" E
5		FRC 25049	11°27'47.0" N; 79°47'45.3" E
7	*R.* × *annamalayana* Kathiresan.	FRC 25052	11°25'55.1" N; 79°48'11.5" E
8		FRC 25053	11°25'45.6" N; 79°48'31.5" E
9		FRC 25054	11°25'33.5" N; 79°48'72.0" E

2.2 Genomic DNA Extraction

Rapidly expanding young leaves from the apical portion of the branch were sampled and dried in silica gel before DNA extraction. *Arboreasy*™ DNA extraction kit (Institute of Forest Genetics and Tree Breeding) was used for extracting genomic DNA using the standard protocol. The purity of the DNA was quantified using a nanodrop spectrophotometer (*nanodrop* Inc., USA).

2.3 PCR Amplification and Microsatellite Genotyping

Fourteen microsatellite markers developed by Shinmura[25] were adopted in this investigation. Eurofins Genomics India Pvt Ltd, Bangalore, India, synthesized the primers. Six of the most polymorphic and repetitive primers (Table 3) were shortlisted based on their banding pattern. A reaction mixture of 10 µl containing 1µl of *Taq* buffer A, 0.3 µl of $MgCl_2$, 0.3 µl of 0.5mM dNTP mix, 0.5 µl of each of the primer, and 1 µl of template DNA (5-10 ng/ul) was subjected to the cyclic thermal amplification using BIO-RAD (BioRad Inc., USA) thermal cycler for 30 cycles. The amplicons were resolved on a 6% Polyacrylamide gel electrophoresis (vertical), and the gels were subjected to silver staining[26]. Resolved gels were photographed using a DSLR camera (Nikon 300, Japan), and the products were scored manually.

3. STATISTICAL ANALYSIS

Co-dominant data was analyzed using *GenAlEx* add-in in Microsoft office excel (2019) [27] to calculate the number of different alleles (*N*.A.), Number of effective alleles (*N*.E.), Observed Heterozygosity (*H*.O.), Expected Heterozygosity (*HE*), Polymorphic Information Content (*PIC*), allelic richness (*R*.S.), and Weight's Index (*FST*)). Phylogenetic analysis of the data sets was done using the Unweighted Pair Group Method with Arithmetic Means (UPGMA) by deploying the software DARwin (6.0.13)[28].

Table 2. Details of *Rhizophora* accession used for the Microsatellite analysis

S. No	Name of the taxon	Tree ID	GBH (cm)	Tree Height (m)	Geo-specifics
1	*R. apiculata*	RA1	27.5	9.0	11°25'73.0" N; 79°47'62.2" E
2		RA2	33.0	12.0	11°26'39.2" N; 79°47'55.8" E
3		RA3	30.5	10.0	11°27'4.70" N; 79°47'45.3" E
4	*R. mucronata*	RM1	35.5	12.0	11°25'55.9" N; 79°47'36.4" E
5		RM2	34.5	11.0	11°27'47.0" N; 79°47'45.3" E
6		RM3	35.5	12.0	11°26'46.3" N; 79°47'54.8" E
7	*R. × annamalayana*	RXA 1	39.0	15.0	11°25'55.1" N; 79°48'11.5" E
8		RXA 2	48.0	12.0	11°25'45.6" N; 79°48'31.5" E
9		RXA 3	37.0	11.0	11°25'33.5" N; 79°48'72.0" E
10		RXA 4	37.5	13.0	11°25'31.8" N; 79°48'81.0" E
11		RXA 5	40.5	14.0	11°25'31.0" N; 79°48'90.0" E
12		RXA 6	35.0	12.0	11°25'28.3" N; 79°48'10.5" E
13		RXA 7	37.5	10.0	11°25'27.2" N; 79°48'98.0" E
14		RXA 8	36.0	12.0	11°25'27.0" N; 79°48'99.0" E
15		RXA 9	35.5	10.0	11°25'26.4" N; 79°48'10.2" E
16		RXA 10	32.5	11.0	11°25'25.4" N; 79°48'10.2" E
17		RXA 11	37.5	11.0	11°25'24.0" N; 79°48'10.6" E
18		RXA 12	31.5	10.0	11°25'22.9" N; 79°48'11.3" E
19		RXA 13	32.0	10.0	11°25'22.9" N; 79°48'11.3" E
20		RXA 14	33.5	7.0	11°25'22.0" N; 79°48'11.5" E
21		RXA 15	36.0	12.0	11°25'55.1" N; 79°48'11.0" E

Table 3. Details of SSR Loci deployed for genotyping studies in *Rhizophora* species complex in Pichavaram, TN.

NCBI accession Number[26]	SSR code		Sequence	SSR motif	*Tm* (C)
AB721972	RM 107	F	GGTTTTCCCAGTCACGACGAACAAGCATGGGCAGGTAAC	$(CT)_{13}$	54
		R	GTTTGCCCATTTGGAATATGTGT		
AB721976	RM 111	F	GGTTTTCCCAGTCACGACGAACCGTTACTCGCGTATGCT	$(T.C.)_{13}$	54
		R	GTTTCATTGCCTCCATTCCATT		
AB721977	RM 112	F	GGTTTTCCCAGTCACGACGTTGAAGGTTGCGGTGAAAT	$(AG)_{13}$	54
		R	GTTTACATTCTTACCCTGCGCACT		
AB721979	RM 114	F	GGTTTTCCCAGTCACGACGATTGGCATAGGCGTTGAATC	$(AT)_{13}$	54
		R	GTTTGTGGCTCAATTGTTGGCTA		
AB721982	RM 121	F	GGTTTTCCCAGTCACGACGTGGCCTATAGAGAAAGCGGA	$(ATC)_{12}$	56
		R	GTTTCCTTCAATCCCAAACAGC		

4. RESULTS

Three in each of the parental species (*R. apiculata* and *R. mucronata*) and fifteen putative hybrids (*Rhizophora* × *annamalayana*) samples were subjected to genotyping using fourteen microsatellite primers[25]. Out of fourteen primers, six Loci were consistent and repetitive across the electrophoresis experiment. These primers were used as a diagnostic tool to understand the variation among the hybrids and their relationship with parental taxa. Microsatellite profile of the loci RM 107 is shown in Figure 1. Descriptive statistics of the *Rhizophora* species complex based on the six polymorphic microsatellite markers are presented in table 4. The average number of alleles (*N*.A.) per locus was 2.0 – 3.3. The loci RM 111 and RM 107 had maximum and minimum alleles, respectively. The Observed Heterozygosity (*H*.O.) and the Expected Heterozygosity (H_E) ranged from 0.444 – 0.867 and 0.473 – 0.647, respectively. The highest expected Heterozygosity (H_E) is associated with the locus RM 114. The allelic richness (*R*.S.) range was 1.90 – 2.84. Polymorphic Information Content (*PIC*) varied from 0.49 – 0.74. The loci RM 112 and RM 116 recorded the highest PICs values of 0.71 and 0.74, respectively. In the present investigation, Weight Index (F_{ST}) values were 0.021 –0.364 and were found statistically significant ($p < 0.05$).

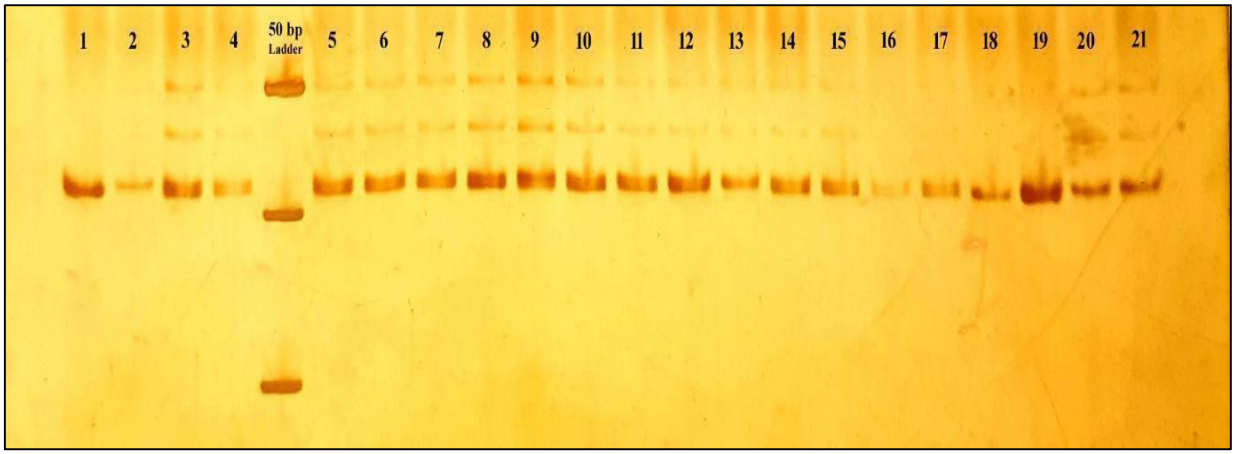

Fig 1: SSR – Microsatellite profile of the loci RM 107 (1, 18 & 19. *R. apiculata*, 2, 20 & 21. *R. mucronata*, 3 – 17. *R. × annamalayana*).

Table 4. Descriptive statistics of different loci used in the study among the *Rhizophora* species complex at Pichavaram, TN.

Name of the Loci	N_A	N_E	H_O	H_E	PIC	F_{SA}
RM 107	3.33	2.394	0.489	0.550	0.66	0.164
RM 111	2.00	1.930	0.600	0.481	0.49	0.021
RM 112	2.33	1.906	0.844	0.473	0.71	0.337
RM 114	3.33	2.847	0.689	0.647	0.70	0.076
RM 116	2.66	2.593	0.444	0.592	0.74	0.203
RM 121	2.33	2.314	0.867	0.553	0.64	0.135
Mean	2.66	2.331	0.656	0.549	0.657	0.156

Table 4 catalogues different measures of genetic diversity among different loci used in the study. Several different alleles (*N*.A.), number of effective alleles (*N*.E.), Observed Heterozygosity (*H*.O.), Expected Heterozygosity (H_E), Polymorphic Information Content (PIC), allelic richness (*R*.S.), and Weight's Index (F_{ST}) the higher the H_E and *PIC* indicate higher diversity.

Table 5 Summary of the genetic diversity at the species level among the *Rhizophora* species complex at Pichavaram, TN.

Name Species	*n*	$N_{A.}$	N_E	H_O	H_E	F_{IS}
R. apiculata	3	2.167	2.100	0.611	0.509	-0.167
R. mucronata	3	2.500	2.300	0.667	0.546	-0.222
R. × annamalayana	15	3.333	2.593	0.689	0.592	-0.190

Table 5 catalogs different measures of genetic diversity among the *Rhizophora* species complex at Pichavaram, Number of individuals (n), Number of different alleles (*N*.A.), number of effective alleles (*N*.E.), Observed Heterozygosity (*H*.O.), Expected Heterozygosity (H_E). Wright's Index or Inbreeding coefficient value (F_{ST}), the higher the H_E and *PIC* indicate higher diversity. The levels of genetic diversity noticed within the species complex among the taxa were found to vary (Table 5). The number of alleles in *R. apiculata*, *R. mucronata*, and *R. × annamalayana* was 2.167, 2.500, and 3.333, respectively. However, the number of effective alleles or allelic richness was more or less similar across species. *R. × annamalayana* had the maximum mean observed heterozygosity (H_E = 0.689), followed by *R. mucronata* (H_E = 0.667) and *R. apiculata* (H_E = 0.611). The expected heterozygosity and Weight Index of *R. apiculata*, *R. mucronata*, and *R. × annamalayana* were 0.509, 0.546, 0.592, and -0.167, -0.222, and -0.190, respectively.

Table 6. Nei's Genetic distances observed between the *Rhizophora* species complex in Pichavaram, TN

	R. apiculata	*R. mucronata*	*R. × annamalayana*
R. apiculata	0.000		
R. mucronata	0.438	0.000	
R. × annamalayana	0.219	0.140	0.000

Nei's genetic distance computed using the allele frequency data among the three species of the *Rhizophora* species complex of Pichavaram is provided in table 6. The values describe the distance and relationship between the analyzed taxa. The phylogenetic relationship based on the UPMGA tree diagram reveals that the putative hybrid is an intermediary to the parental species (Fig 2). Nei's distance between *R. apiculata* and *R. × annamalayana* was recorded to be 0.219, while the distance between *R. mucronata* and *R. × annamalayana* was 0.140. Further, the distance between the parental species (*R. apiculata* and *R. mucronata*) was 0.438. The parental species *R. apiculata* and *R. mucronata* are in the north and south of the dendrogram, and the putative hybrid genotypes fit in between. The hybrid accessions R×A-1 to R×A 10 clustered with *R.*

mucronata, whereas accessions R×A-11 to R×A-15 were grouped with *R. apiculata*. Nei's genetic distances (Table 6) observed among the species indicate that the putative hybrid species is genetically more proximal to *R. apiculata* than *R. mucronate*.

5. DISCUSSION

Mangroves are potential forest genetic resources with enormous phyto-pharmacological values. However, these valuable resources are severely threatened due to anthropogenic and natural pressures [29,30]. The decrease in the efficacy of antibiotics owing to predisposition has accelerated demand for alternative drugs[31]. Hence, effective strategies to manage genetic diversity in Forest Genetic Resources gain paramount importance. Studies on biotically pollinated and abiotically seed dispersed taxa such as *Ceriops*, *Kandelia* and *Bruguiera* show low genetic variations [32,33]. However, Rhizophora, an abiotically pollinated and dispersed genus, is of concern. Earlier studies in the Pichavaram indicate low levels of genetic variation within the population among species [15,16]. This investigation reveals that the putative hybrid *R.* × *annamalayana* (mean H_E = 0.592) has more significant variability than its putative parents *R. apiculata* (mean H_E 0.611) and *R. mucronata* (mean H_E = 0.667). The genetic diversity values noted among the Pichavaram hybrid populations are higher than that of *R. mucronata* (mean H_E = 0.354) and *R. stylosa* (mean H_E = 0.321) populations in Malaysia[34]. The negative inbreeding coefficient value or Wright's Index in *R.* × *annamalayana* (- 0.190) suggests ample variation within the putative hybrids. This could be because of the varying mating systems among the parental species. The observed genetic distances are consistent with the earlier study in site [10] using the dominant marker system. Ten samples out of fifteen clustered with *R. mucronata*, and the rest five grouped with *R. apiculata*, indicating the varying paternal and maternal combinations. Further, investigations on Chloroplast DNA and Mitochondrial DNA on these lines may present more insights. The hybrid *R.* × *annamalayana* draws its lineage from both the putative parents. Both the putative parents of the hybrid share the common niche and overlap in the phenology leading to the natural hybridization[19,35]. The taxon *R. apiculata* is reportedly threatened in Southeast Asia due to overexploitation of the tannin resources [36]. Similarly, *R. apiculata* in the study location also had less population size. Notably, the loss of genetic diversity in one of the putative parents could implicate the process of natural hybridization. Hence, genetic diversity management during rehabilitation could amplify their rate of success. Further studies on this aspect with more markers and samples would give better insights into understanding the adaptive variations of the *Rhizophora* species complex in Pichavaram. This study demonstrates significant variations within the *R.* × *annamalayana* samples at an intra-population level. This suggests that the ecosystem has generated genomically, significantly varying recombinants. The putative hybrid population in this location needs further characterization and conservation at the forest genetic resource level. Conventional breeding technologies could be adapted to generate intraspecific hybrids with pedigrees, perhaps this could aid in improving the hybrid population and create an avenue for tree breeding research on developing wide tannin-yielding varieties if appropriate tree breeding and (or) improvement approaches are deployed.

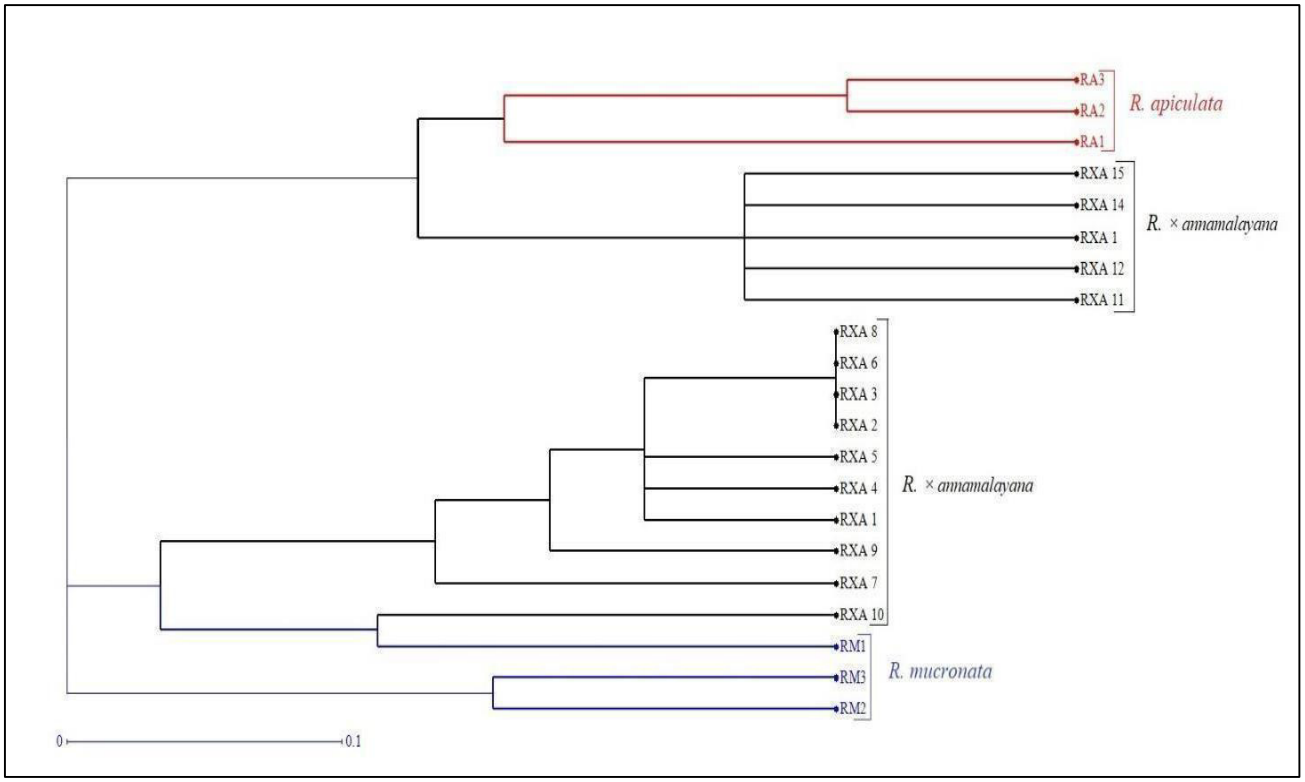

Fig 2. Dendrogram showing the genetic relationship of the Hybrid *R.* × *annamalayana* with its parental species

6. CONCLUSION

The study indicates high genetic diversity among the *Rhizophora* species complex in Pichavaram. This suggests that the ecosystem has generated genomically, significantly varying recombinants. The putative hybrid population in this location needs further characterization and conservation at the forest genetic resource level.

7. ACKNOWLEDGEMENTS

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8. AUTHORS CONTRIBUTION STATEMENT

Dr. B. Nagarajan hypothesized the study and provided valuable inputs during the manuscript preparation. Dr. A. Shanthi, designed the primers and offered practical guidance during lab research and manuscript preparation. Mr. M. Utchimahali conducted the experiment, gathered data and drafted the manuscript. Ms. P. Maheshwari, Mr. K. Nithishkumar and S. Haritha supported in gathering wet lab data and analysis.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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